Diversity in the CDR3 Region of V_H Is Sufficient for Most Antibody Specificities

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Summary

All rearranging antigen receptor genes have one or two highly diverse complementarity determining regions (CDRs) among the six that typically form the ligand binding surface. We report here that, in the case of antibodies, diversity at one of these regions, CDR3 of the V_H domain, is sufficient to permit otherwise identical IgM molecules to distinguish between a variety of hapten and protein antigens. Furthermore, we find that somatic mutation can allow such antibodies to achieve surprisingly high affinities. These results are consistent with a model in which the highly diverse CDR3 loops are the key determinant of specificity in antigen recognition in both T cell receptors (TCR) and antibodies, whereas the germline-encoded CDR1 and CDR2 sequences are much more cross-reactive.

Introduction

During lymphocyte development, a large repertoire of heterodimeric antigen receptors, both antibodies and TCRs, are generated by a variety of mechanisms (Tonegawa, 1983; Davis and Bjorkman, 1988). In antibodies, the binding site for antigens is formed by six CDRs that loop out from the V region backbone formed by two sheets of β-pleated strands (reviewed in Davies et al., 1990). Great importance has been attached to the germline V gene repertoire for the development of effective immune responses, as most of the CDRs are encoded by the germline sequences (with the exception of CDR3 of the heavy chain). It has been postulated that the V region genes are selectively retained in the germline during evolution because of their capacity to accommodate different antigens, especially pathogens (e.g., Cohn et al., 1980; Rajewsky et al., 1987).

Recent structural analyses show that in αβ TCRs, amino acids at positions equivalent to the CDRs in antibodies also form the principal contacts between TCRs and their peptide/MHC ligands (Garboczi et al., 1996; Garcia et al., 1996; Ding et al., 1998; Reinherz et al., 1999). While no complete structure of a γδ TCR in complex with its ligand is currently available, a number of studies indicate that γδ TCRs recognize antigens in an antibody-like manner (reviewed in Chien et al., 1996; see also Li et al., 1998). The binding interfaces between antigen receptor molecules and their ligands are generally large (over 1500 Å^2) and 10–30 side chains from each side make close contacts (reviewed in Davies et al.,

1990; Davies and Cohen, 1996; Garcia et al., 1999). However, despite the broad interface seen in the crystal structures, sequence diversity in antigen receptors is not evenly distributed among all six CDRs but is highly concentrated in one (in the cases of antibodies and γδ TCRs) or two (in the case of αβ TCRs) CDR3s (Davis and Bjorkman, 1988; Davis and Chien, 1999). Although the skewing of diversity toward CDR3s in αβ TCRs is understandable because these amino acid residues mainly recognize the antigenic peptide while other CDRs primarily interact with MHC (Jorgensen et al., 1992; Garboczi et al., 1996; Garcia et al., 1996; Sant'Angelo et al., 1996; Ding et al., 1998; Reinherz et al., 1999), there has been no explanation for such a phenomenon in antibodies and γδ TCRs. Even more puzzling is the finding that in antibodies, both CDR1 and CDR2 of the heavy chains and all CDRs of the light chains have only a few "canonical" conformations, and only the heavy chain CDR3 loop has a wide range of variations in both length and shape (Chothia and Lesk, 1987; Chothia et al., 1989). Such canonical CDR loop shapes have also been seen in αβ TCR crystal structures (CDR2 of α and β particularly, but not in CDR3α or CDR3β) (Garcia et al., 1999), although some of this uniformity may relate to MHC binding requirements. The skewing of diversity in all antigen receptor molecules has led to the suggestion that the highly diverse CDR3 sequences are the principal determinant of specificity in antigen recognition, at least in the primary repertoire (Davis et al., 1997, 1998).

To further understand the molecular basis of antigen receptor specificity, we have constrained mice to use a single V_H gene but full CDR3 diversity to generate their B cell repertoire. We challenged such mice with a variety of protein and hapten antigens and monitored the development of primary and memory immune responses. We find that antigen-specific IgM molecules isolated from primary response of these mice can differ only in the CDR3 of the V_H domain; furthermore, we find that somatic mutation can allow such antibodies to achieve surprisingly high affinities upon rechallenge with protein antigens. The only antigens that seem generally unable to be accommodated by an arbitrarily chosen V_H are bacterial polysaccharides. These results indicate that an extensive V (both V_H and V_L) gene repertoire is not necessary for the production of specific antibodies to most antigens, and that the V_H CDR3 plays a very different role in the makeup of the antibody binding site than the germline-encoded CDR1 and CDR2 sequences. These results suggest that the purpose of the highly diverse CDR3 region of all antigen receptors, both TCRs and antibodies, is to determine antigen specificity.
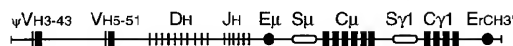
Results

Experimental System

In order to characterize the antibody responses of an organism with a very limited V region repertoire but full

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A. Heavy Chain Minilocus, HC1

ψVH3-43   VH5-51   DH   JH   Eμ Sμ   Cμ   Sγ1 Cγ1 ErCH3'

B. λ Light Chain Locus

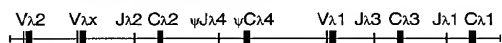Vλ2   Vλx   Jλ2 Cλ2 ψJλ4 ψCλ4   Vλ1 Jλ3 Cλ3 Jλ1 Cλ1

Figure 1. Physical Map of the Transgenic Heavy Chain Minilocus *HC1* and the Endogenous Mouse λ Light Chain Locus

Drawings are schematic and not to scale. ψ denotes pseudogenes. V, variable gene segment; D, diversity segment; J, joining segment; C, constant region gene; S, switch region; E, enhancer.

CDR3 diversity, we utilize a previously described trans-gene, *HC1*, which contains one functional human V_H gene segment (V_H5–51), ten functional D segments, six J_H segments, and the C_μ and $C_\gamma 1$ constant regions (Figure 1A; Taylor et al., 1992, 1994). The *HC1* transgenic

mice were bred onto an *IgH*$^{-/-}$ *Ig*κ$^{-/-}$ background (Chen et al., 1993a, 1993b). With this genotype (*HC1*$^{+/0}$ *IgH*$^{-/-}$ *Ig*κ$^{-/-}$), the only functional immunoglobulin that can be produced would combine the single V_H5–51 with one of the three λ light chain V regions (V_λ1, V_λ2, or V_λx; Figure 1B) from the mouse. Flow cytometry analysis revealed that the *HC1* transgene partially rescues the B cell compartment of an *IgH*$^{-/-}$ *Ig*κ$^{-/-}$ mouse, although B cell numbers are extremely low (5%–10% normal; data not shown). Serum analysis shows that levels of IgM and IgG1 range from 60 to 300 μg/ml (5%–10% normal) and 0.3 to 5.0 μg/ml (0.1%–1% normal), respectively, in these mice (data not shown).

Antigen-Specific Development of Immune Responses in the *HC1*$^{+/0}$ *IgH*$^{-/-}$ *Ig*κ$^{-/-}$ Mice

To test whether the B cell repertoire with diversity only in V_H CDR3 is effective, we immunized the *HC1*$^{+/0}$ *IgH*$^{-/-}$ *Ig*κ$^{-/-}$ mice with a variety of protein antigens: keyhole limpet hemocyanin (KLH), hen egg-white lysozyme (HEL),
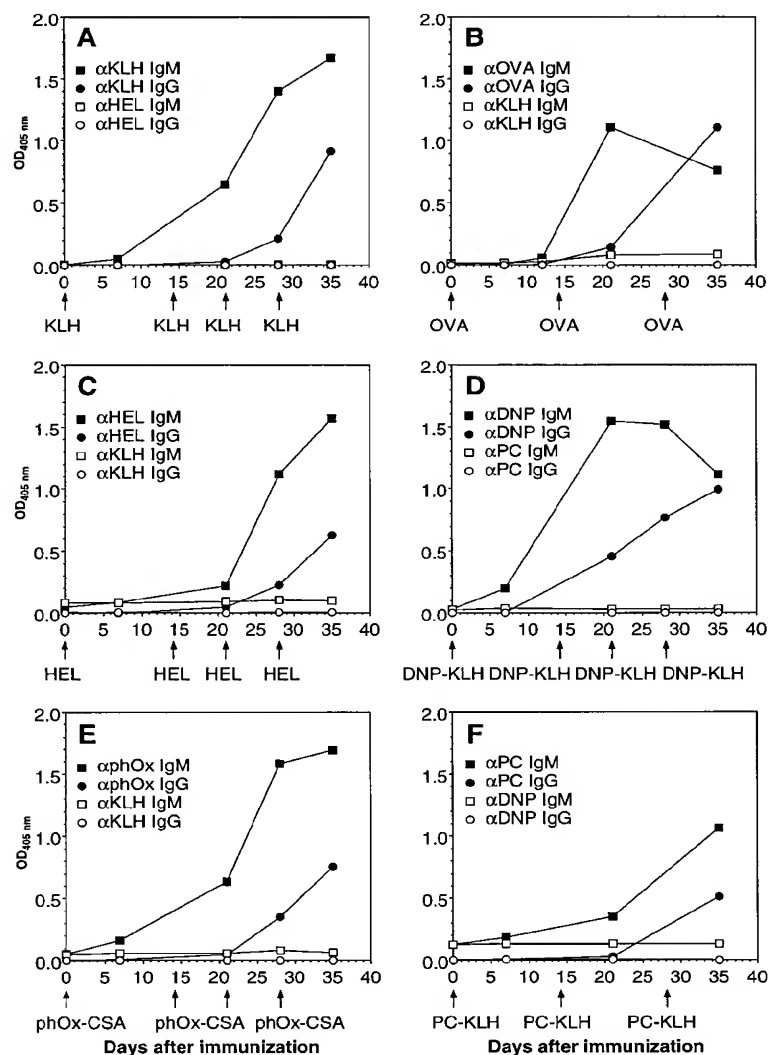

Figure 2. Antigen-Specific Development of IgM and IgG Responses in the *HC1*$^{+/0}$ *IgH*$^{-/-}$ *Ig*κ$^{-/-}$ Mice

The *HC1*$^{+/0}$ *IgH*$^{-/-}$ *Ig*κ$^{-/-}$ mice were immunized with KLH (A), OVA (B), HEL (C), DNP-KLH (D), phOx-CSA (E), or PC-KLH (F) on indicated days. Serum samples were collected 7 days after each injection and diluted 1:10–1:250 into microtiter wells coated with KLH or HEL (A), OVA or KLH (B), HEL or KLH (C), DNP-BSA or PC-BSA (D), phOx-BSA or KLH (E), or PC-BSA or DNP-BSA (F). Antigen-bound IgM or IgG was detected as described in the Experimental Procedures. At least three mice were tested for each antigen and for clarity, results from a representative mouse were shown. Variations among individual mice were within a 5-fold range.

A

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VH5-51:   EVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWTGWVRQMPGKGLEWMGIIYPGDSDTRYSPSFQGQVTISADKSISTAYLQWSSLKASDTAMYYCAR_____CDR3_____
DNP-6:    --------------------------------------------------------------------------------------------------QIPS      YWYFDLWGRG
DNP-7:    --------------------------------------------------------------------------------------------------QTEG      YWYFDLWGRG
DNP-8:    --------------------------------------------------------------------------------------------------QKPH      YWYFDLWGRG
DNP-10:   --------------------------------------------------------------------------------------------------VLNWV     FDYWGQG
DNP-11:   --------------------------------------------------------------------------------------------------QAFS      YWYFDLWGRG
DNP-12:   --------------------------------------------------------------------------------------------------HGAR      YWYFDLWGRG
KLH-1:    -----------------------I----------------------------------------------------------------------------GLLWFGE   FDYWGQG
KLH-3:    -----------------------T----------------------------------------------------------------------------NLLD      AFDIWGQG
KLH-5:    --------------------------------------------------------------------------------------------------HEWFGEL   DYWGQG
KLH-6:    --------------       .----------------------------------------------------------------------------SDSSSWYWGSF FDYWGQG
OVA-1:    --------------------------------------------------------------------------------------------------FRLGMT    FDIWGQG
OVA-2:    --------------------------------------------------------------------------------------------------FKLGMT    FDIWGQG
OVA-4:    --------------------------------------------------------------------------------------------------HRM       YYYGMDVWGQG
OVA-5:    --------------------------------------------------------------------------------------------------WRYFDWFL  FDYWGQG
OVA-6:    --------------------------------------------------------------------------------------------------QRITMVRGGMG FDYWGQG
HEL-1:    --------------------------------------------------------------------------------------------------HVRSSSWYF YYGMDVWGQG
HEL-2:    --------------------------------------------------------------------------------------------------VTGDRD    YYGMDVWGQG
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B

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VΛ1:      QAVVTQESALTTSPGETVTLTCRSSTGAVTTSNYANWVQEKPDHLFTGLIGGTNNRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFCALWYSNH WVFGGGTKLTVLG
VΛ2:      --------------G-----.---------------------------S--------V---------------------D-M--------T-      Y-------V----
DNP-6:    -----------------------------------------------------------------------------------------------   L------------
DNP-7:    -----------------------------------------------------------------------------------------------   L------------
DNP-8:    -----------------------------------------------------------------------------------------------   L------------
DNP-10:   ---------------------------------------------------------------------------------------...         .-----
DNP-11:   -----------------------------------------------------------------------------------------------   L------------
DNP-12:   -----------------------------------------------------------------------------------------------   L------------
KLH-1:    -----------------------------------------------------------------------------------------------   F------------
KLH-3:    -----------------------------------------------------------------------------------------------F------------
KLH-5:    -----------------------------------------------------------------------------------------------   L------------
KLH-6:    -----------------------------------------------------------------------------------------------   -------------
OVA-1:    -----------------------------------------------------------------------------------------------   L------------
OVA-2:    -----------------------------------------------------------------------------------------------   L------------
OVA-4:    -----------------------------------------------------------------------------------------------   L------------
OVA-5:    -----------------------------------------------------------------------------------------------   F------------
OVA-6:    -----------------------------------------------------------------------------------------------   -------------
HEL-1:    -----------------------------------------------------------------------------------------------   -------------
HEL-2:    -----------------------------------------------------------------------T-      Y-------V----
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Figure 3. Sequence Analysis of Monoclonal Anti-DNP, Anti-OVA, Anti-KLH, and Anti-HEL IgM Antibodies Originated from the $HC1^{+/0}$ $IgH^{-/-}$ $Ig\kappa^{-/-}$ Mice

The cDNA encoding monoclonal antibodies against various antigens was synthesized and amplified by RT-PCR, cloned, and sequenced. Shown are the deduced amino acid sequences of both heavy (A) and light (B) chains of various monoclonal antibodies. The top line in (A) is the germline V_H5–51 sequence; the top two lines in (B) are the germline $V_\lambda1$ and $V_\lambda2$ sequences. Dashes in the sequences indicate identity to the top line. The CDRs in both heavy and light chain are underlined. In V_H CDR3, amino acid residues encoded by the J segment are separated by spaces from those encoded by the D segment (in bold) and N nucleotides. D segment assignments are based on at least six nucleotides of homology; amino acid residues not accounted for by D are presumably encoded by the N nucleotides. All monoclonal antibodies shown here are of IgM isotype. Some of these hybridomas (e.g., DNP-8, DNP-11, KLH-1, KLH-3, and OVA-2) were isolated twice from the same fusion.

chicken ovalbumin (OVA), cholera toxin B subunit (CTB), as well as haptens: 2,4-dinitrophenyl (DNP, conjugated to KLH), 2-phenyl-4-ethoxymethylene-5-oxazolone (phOx, conjugated to chicken serum albumin, CSA), and phosphorylcholine (PC, conjugated to KLH). Figure 2 shows the results of enzyme-linked immunosorbent assay (ELISA) of some of these responses. As typical of normal immune responses, IgM antibodies appeared first, followed by IgG. All *HC1* mice examined responded to all antigens tested, except the pigeon cytochrome c (PCC) protein, to which only three out of nine mice were able to make a detectable response (data not shown), suggesting that B cell clones with this specificity are very rare in these mice.

Sequence Analysis of Antigen-Specific Antibodies Derived from the $HC1^{+/0}$ $IgH^{-/-}$ $Ig\kappa^{-/-}$ Mice

It has been previously reported that in mice with a fixed VDJ_H rearrangement, V gene replacement and somatic hypermutation can generate a significant level of functional diversity in the primary repertoire (Cascalho et al., 1996; López-Macías et al., 1999). It was therefore possible that secondary rearrangements and/or somatic mutations could contribute to the antibody responses seen in the $HC1^{+/0}$ $IgH^{-/-}$ $Ig\kappa^{-/-}$ mice. To control for this, as well as to characterize the responding antibodies in

more detail, we immunized $HC1^{+/0}$ $IgH^{-/-}$ $Ig\kappa^{-/-}$ mice with antigens and isolated hybridoma cell lines producing antigen-specific IgM antibodies. Animals were immunized only once to minimize somatic hypermutation. Complementary DNA (cDNA) from both heavy and light chain mRNA of these antibodies was synthesized, amplified by polymerase chain reaction (PCR), and then cloned and sequenced. All the hybridomas characterized used both V_H5–51 and C_μ from the *HC1* transgene in their heavy chain and used either $V_\lambda1$–$J_\lambda1$ or $V_\lambda2$–$J_\lambda2$ in their light chain (with the exception of hybridoma HEL-2, which appears to use $V_\lambda1$–$J_\lambda2$ in its light chain; Figure 3 and data not shown). No $V_\lambda x$ usage was observed.

A comparison of the anti-DNP, anti-KLH, anti-OVA, and anti-HEL IgM sequences shows that most express the V_H5–51 gene segment in its germline form and thus differ only in the CDR3 region of the heavy chain. In addition, there are two large sets of hybridomas that express identical light chains as well. The first set includes DNP-6, DNP-7, DNP-8, DNP-11, DNP-12, KLH-5, OVA-1, OVA-2, and OVA-4; and the second includes DNP-10, KLH-6, OVA-6, and HEL-1 (Figure 3). ELISA assays on all of these antibodies confirmed that they only bind the antigen they were raised against, consistent with the whole serum analysis shown in Figure 2

A

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VH5-51: EVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWTGWVRQMPGKGLEWMGIIYPGDSDTRYSPSFQGQVTISADKSISTAYLQWSSLKASDTAMYYCAR_____CDR3_____
OVA-7:  -----------------------T--R---A------------------G-----------------V-------------------F---HWGAY YYYYYGMDVWGQG
HEL-3:  ----E----------------------------------------D-------------------------------V-LSGD       WFDPWGQG
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B

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Vλ1:    QAVVTQESALTTSPGETVTLTCRSSTGAVTTSNYANWVQEKPDHLFTGLIGGTNNRAPGVPARFSGSLIGDKAALTITGAQTEDEAIYFCALWYSNH  WVFGGGTKLTVLG
OVA-7:  --------T------------------T--V-------S-V-A----H---------------------V-----------------  L-----------
HEL-3:  -T-------------------N--S-SP-------------A---D------------------------------------K-INT------------
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Figure 4. Sequence Analysis of Monoclonal Anti-OVA and Anti-HEL IgG1 Antibodies Originated from the $HC1^{+/0}$ $IgH^{-/-}$ $Ig\kappa^{-/-}$ Mice

The cDNA encoding monoclonal IgG1 antibodies against OVA (OVA-7) and HEL (HEL-3) was synthesized, amplified by RT-PCR, cloned, and sequenced. Shown are the deduced amino acid sequences. Similar as in Figure 3, D segment assignments are based on at least six nucleotides of homology.

(data not shown). These results show that diversity in the CDR3 region of V_H alone is sufficient to obtain highly specific IgM antibodies to these antigens.

Among the DNP-specific hybridomas isolated, two sequences (DNP-8 and DNP-11) were seen twice; in addition, DNP-6 and DNP-11 are different at only one position (isoleucine versus alanine) in V_H CDR3. Furthermore, the junctional diversity in the heavy chain is restricted. For example, all but one hybridomas had identical CDR3 length, utilized J_H2 gene segment, and expressed glutamine at position 95 (the first amino acid in the junctional region); in the light chains, all but one had leucine at position 96 (the first amino acid in the junctional region), even though tryptophane is encoded at that position in the germline $J_\lambda1$. In the hybridomas specific for protein antigens, there were identical repeats as well: for example, sequences represented by KLH-1, KLH-3, and OVA-2 were all isolated twice. Also, OVA-1 and OVA-2 are different only at position 96 in the heavy chain, with arginine in OVA-1 and lysine in OVA-2; both arginine and lysine residues are positively charged.

High-Affinity IgG1 Antibodies for Protein Antigens from the $HC1^{+/0}$ $IgH^{-/-}$ $Ig\kappa^{-/-}$ Mice

Having established that the $HC1^{+/0}$ $IgH^{-/-}$ $Ig\kappa^{-/-}$ mice are capable of mounting highly specific antibody responses to most, if not all, of the antigens, we asked whether such mice can produce high-affinity antibodies. We pursued this question both by generating monoclonal IgG antibodies through hyperimmunization and by purifying polyclonal IgG antibodies from antisera. We obtained two IgG1 monoclonal antibodies, one specific for HEL (HEL-3) and the other for OVA (OVA-7). Both monoclonal antibodies used the $V_H5-51-C_\gamma1$ in the heavy chain and $V_\lambda1-J_\lambda1-C_\lambda1$ in the light chain. Both HEL-3 and OVA-7 antibodies also exhibit evidence of somatic hypermutation in both the $\gamma1$ and λ chains with most of the mutations being located in the CDR regions (Figure 4), as is typical.

We then employed surface plasmon resonance (SPR) technique (Malmqvist, 1993) to measure the affinity of these antibodies. We immobilized the HEL-3 antibody on the biosensor chip and passed different concentrations of HEL over the immobilized antibody (Figure 5A). Fitting of the binding curves gives an association rate of 9.8×10^6 M^{-1} s^{-1} and a dissociation rate of 2.5×10^{-4} s^{-1} at 25°C. These values give a K_D of 2.55×10^{-11} M, or 25.5 pM, equivalent to the highest affinity anti-HEL antibodies known (Davies and Cohen, 1996). For unknown reasons, immobilization of OVA-7 on a biosensor chip leads to its inactivation (data not shown); thus, we immobilized OVA on the biosensor chip and allowed OVA-7 to bind to the immobilized ligand (Figure 5B). While accurate fitting of the bivalent IgG binding is not
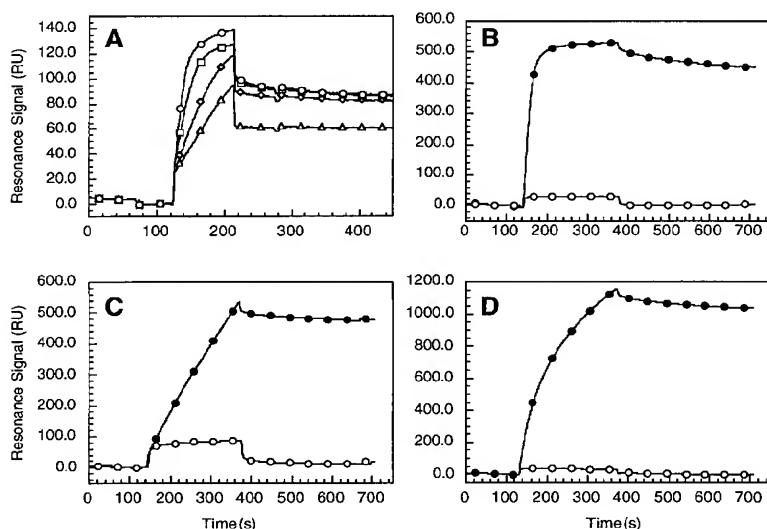

Figure 5. High-Affinity IgG1 Antibodies Derived from the $HC1^{+/0}$ $IgH^{-/-}$ $Ig\kappa^{-/-}$ Mice

(A) HEL was passed over the immobilized monoclonal HEL-3 antibody (250 RU) at concentration of 2.5 (triangles), 5.0 (diamonds), 10 (squares), and 20 (circles) nM at a flow rate of 100 μl/min.

(B) OVA-7 (20 μg/ml) was passed over immobilized OVA (1000 RU, closed circles) or empty surface (open circles).

(C-D) $HC1^{+/0}$ $IgH^{-/-}$ $Ig\kappa^{-/-}$ mice were immunized with HEL (C) or OVA (D). Antiserum samples were collected after fourth, fifth, and sixth injection and pooled. Total IgG1 was purified from preimmune (open circles) or antiserum (closed circles) with affinity column using goat anti-human IgG1 (Fc specific) antibody conjugated to agarose beads, and passed over HEL (C) or OVA (D) coupled to the CM5 sensor chip at a concentration of 25 μg/ml.

possible, we did find that OVA-7 dissociates very slowly: the apparent dissociation rate is $\sim 5.0 \times 10^{-4}$ s^{-1}. As bivalent antibodies have dissociation rates that are approximately 10-fold slower than their monovalent Fab fragments (Mason and Williams, 1986), the estimated bona fide dissociation rate constant of OVA-7 is approximately 5.0×10^{-3} s^{-1}. Given a typical association rate of $0.5^{-5} \times 106$ M^{-1} s^{-1} for protein–protein interactions (Northrup and Erickson, 1992), the K_D of OVA-7 would be in the 1–10 nM range, comparable with monoclonal antibodies isolated from normal mice.

Total IgG1 antibodies from the $HC1^{+/0}$ $IgH^{-/-}$ $Ig\kappa^{-/-}$ mice immunized with HEL, OVA, or KLH were also used in SPR analysis (Figures 5C and 5D and data not shown). Again, we found that polyclonal IgG1 antibodies purified from the $HC1^{+/0}$ $IgH^{-/-}$ $Ig\kappa^{-/-}$ mice bind their cognate antigen fairly tightly, with an apparent dissociation rate of $\sim 1 \times 10^{-4}$ s^{-1} (Figures 5C and 5D). Thus, the average K_D of these antibodies would also be in the 0.2–2 nM range, assuming a typical association rate of 0.5–5 $\times$ 10^6 M^{-1} s^{-1}.

Taken together, the antigen binding data from both HEL-3 and OVA-7 monoclonals and the polyclonal antisera shows that somatic mutation can allow antibodies from the $HC1^{+/0}$ $IgH^{-/-}$ $Ig\kappa^{-/-}$ mice to achieve very high affinities against at least protein antigens, comparable to mice with a full V region repertoire.

Failure of the $HC1^{+/0}$ $IgH^{-/-}$ $Ig\kappa^{-/-}$ Mice to Respond to Bacterial Polysaccharides

If one V_H gene is able to accommodate most or all of the antigens, why are there so many different V_H genes in most vertebrate genomes? It is currently thought that the T cell–independent IgM response to bacterial polysaccharides provides an important protection to the host during infections (Janeway and Travers, 1997). If this defense relies on a large V region repertoire, as previously suggested (Cohn et al., 1980; Rajewsky et al., 1987), the $HC1^{+/0}$ $IgH^{-/-}$ $Ig\kappa^{-/-}$ mice may not be able to mount an antibody response to some bacterial carbohydrate antigens. Therefore, we injected five $HC1^{+/0}$ $IgH^{-/-}$ $Ig\kappa^{-/-}$ and five C57BL/6 (B6) mice with dextran B512 (DEX) from *Leuconostoc mesenteroides*, a near-linear glucose polymer with 96% $\alpha(1{\rightarrow}6)$ linkages, and followed the immune response by ELISA. DEX-specific IgM was detected in every B6 mice after the first injection (Figure 6B); however, no IgM response was observed in any of the $HC1^{+/0}$ $IgH^{-/-}$ $Ig\kappa^{-/-}$ mice even after three injections of DEX (Figure 6A). The $HC1^{+/0}$ $IgH^{-/-}$ $Ig\kappa^{-/-}$ mice also failed to respond to levan, a branched fructose polymer from *Erwinia herbicola* (Figures 6C and 6D). In contrast, the $HC1^{+/0}$ $IgH^{-/-}$ $Ig\kappa^{-/-}$ (as well as B6) mice responded to other T-independent antigen, for example, 2,4,6-trinitrophenyl (TNP)-Ficoll (Figures 6E and 6F). Thus, the lack of responsiveness to the two polysaccharide antigens in the $HC1^{+/0}$ $IgH^{-/-}$ $Ig\kappa^{-/-}$ mice is probably not due to lack of T cell help. Since all anti-DEX monoclonal antibodies reported use the κ light chain (Akolkar et al., 1987; Wang et al., 1990), and it has been shown that the $V\kappa$ domains do provide antigenic contacts in these antibodies (Wallick et al., 1989), it is possible that the failure of the $HC1^{+/0}$ $IgH^{-/-}$ $Ig\kappa^{-/-}$ mice to respond to DEX could be solely due to the lack of the appropriate
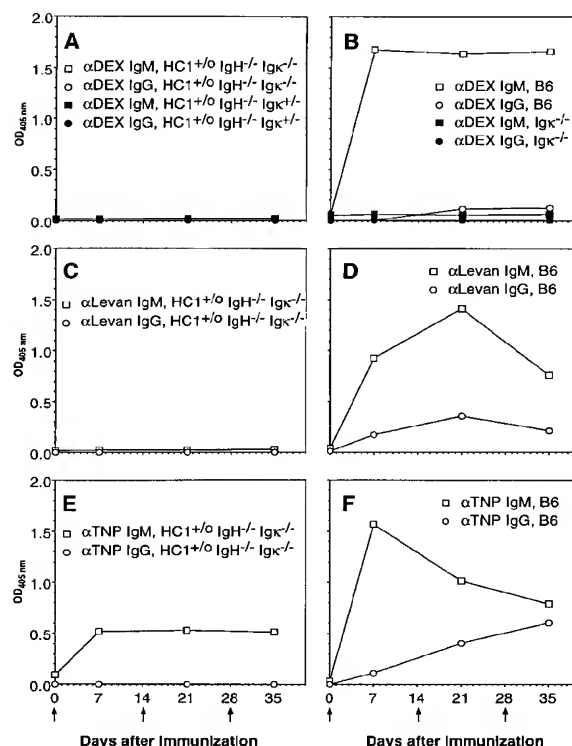

Figure 6. $HC1^{+/0}$ $IgH^{-/-}$ $Ig\kappa^{-/-}$ Mice Mount IgM Response to TNP-Ficoll but Not to DEX or Levan

Mice with indicated genotypes were injected intravenously with 10 μg DEX (A and B) or TNP-Ficoll (E and F), or intraperitoneally with 50 μg levan (C and D) on day 0, 14, and 28. Serum samples were collected 7 days after each injection and diluted 1:10–1:250 into microtiter wells coated with DEX (A and B), levan (C and D), or TNP-BSA (E and F). Antigen-binding human or mouse antibodies (IgM and IgG) were detected with alkaline phosphatase conjugated goat anti-human or goat anti-mouse secondary antibodies.

$V\kappa$ chain(s). To test this possibility, we reintroduced a functional $Ig\kappa$ locus into the mutant mice, but the resulting animals ($HC1^{+/0}$ $IgH^{-/-}$ $Ig\kappa^{+/-}$) were still unable to respond to DEX (Figure 6A). In addition, we also immunized $Ig\kappa^{-/-}$ mice (with a normal V_H gene repertoire) with this antigen, and they were also unable to respond (Figure 6B).

The observation that ($IgH^{+/+}$) $Ig\kappa^{-/-}$, $HC1^{+/0}$ $IgH^{-/-}$ $Ig\kappa^{+/-}$, and $HC1^{+/0}$ $IgH^{-/-}$ $Ig\kappa^{-/-}$ mice all failed to respond to DEX indicates that neither V_H5–51 nor any of the V_λ genes can be utilized to accommodate this antigen. However, it cannot be ruled out that a very low frequency of DEX-specific B cells were still present in these mice, but the few precursor B cells were overwhelmed by an excess of antigen (i.e., high zone tolerance was induced) (Howard et al., 1975; Fernandez and Möller, 1977).

Discussion

It has been known for some time that in both B and T cell recognition, some epitopes show restricted V gene usage. For example, very limited V_H and V_L genes are

used in immune responses of inbred mice to many hapten, polysaccharide, and even protein antigens, including p-azophenylarsonate (Pawlak et al., 1973; Mäkelä et al., 1976; Manser, 1990), phenyloxazolone (Mäkelä et al., 1978; Kaartinen et al., 1983; Berek and Milstein, 1987), (4-hydroxy-3-nitrophenyl)acetyl (NP) (Imanishi and Mäkelä, 1974, 1975; Blier and Bothwell, 1988), DNP (Dzierzak et al., 1980, 1985), phosphorylcholine (Potter and Lieberman, 1970; Sher and Cohn, 1972; Barstad et al., 1974; Crews et al., 1981), phosphatidylcholine (Seidl et al., 1997), dextran (Blomberg et al., 1972; Riblet et al., 1975; Wang et al., 1990), galactan (Mushinski and Potter, 1978), Staphylococcal nuclease (Fathman et al., 1977), and influenza hemagglutinin (McKean et al., 1984; Clarke et al., 1985). T cell recognition of peptide/MHC complexes is often (but not always) fairly restricted with respect to V gene usage (e.g., Hedrick et al., 1988). However, it is not clear whether the preferred V gene segment(s) are absolutely required for such epitopes or are merely the result of extensive clonal expansion where a slight advantage in binding would allow the most optimal $V_H V_L$ combination to predominate. In the current study, we find that mice having only one functional V_H and (effectively) two V_L gene segments are able to mount highly specific antibody responses to most antigens. It thus seems likely that the restricted V gene usage observed in most immune responses represents the consequence of antigen-driven clonal expansion of kinetically favorable antigen receptor clone(s).

In the $HC1^{+/0}$ $IgH^{-/-}$ $Ig\kappa^{-/-}$ mice, we find a remarkable restriction in V_H CDR3 diversity with respect to both length and amino acid composition in the primary immune response to both hapten and protein antigens. In the IgM antibodies, there are many identical or very similar sequences. A restrictive pattern of V_H CDR3 would normally be interpreted to mean antigen-driven clonal expansion; however, considering the low efficiency of cell fusion, the isolation of identical repeats suggests that certain antibody specificities could be specifically expanded even prior to immunization (Rajewsky et al., 1987). It is not clear at present whether the expansion of a particular specificity occurs as a result of preferred V(D)J rearrangement event or is the result of some type of selection.

Our results also indicate that the antigen binding site of nonsomatically mutated antibodies is not uniformly specific but instead consists of one highly diverse CDR loop (V_H CDR3) and at least four "generic" antigen binding regions (CDRs 1+2). Even the somewhat diverse V_L CDR3 region seems to play a very minor role. Using a phage display library, Winter and colleagues previously showed that a range of hapten and protein binding activities could be isolated from a repertoire of antibodies comprising 50 human V_H gene segments in combination with a fixed light chain (Hoogenboom and Winter, 1992; Nissim et al., 1994). That V_H CDR3 might play a dominant role in antigen binding was also suggested by earlier studies showing that a heavy chain alone or even single V_H domains can bind antigens with a comparable affinity as the intact antibody (Ward et al., 1989; Noël et al., 1996). In addition, it has been found that randomly mutagenizing the V_H CDR3 of an anti-tetanus toxin antibody enables the isolation of a new specificity (i.e., for fluorescein) (Barbas et al., 1992). Our results are also consistent

with the findings that murine B cells that have a rearranged heavy chain immunoglobulin gene in the germline always change the CDR3 sequence and often the V_H as well when they deviate from the original antigen reactivity (Cascalho et al., 1996; López-Macías et al., 1999). Our data may explain the long-standing puzzle of "canonical" CDR structures in antibodies (Chothia and Lesk, 1987; Chothia et al., 1989). We would suggest that uniform CDR shapes are important for the stability of a binding site, with the exception of V_H CDR3, which has to "fit" the antigen surface much more precisely. These results are also reminiscent of the case of human growth hormone and its receptor, where structural analysis shows that about 30 side chains from each protein are in close contact but only a fraction of those residues have any contribution to the binding affinity and only two account for more than three-quarters of the free energy (Wells, 1996).

Another interesting issue raised by the data presented here is the role of somatic mutation in producing higher-affinity antibodies upon rechallenge with antigen. Our data shows that, at least for protein antigens, having an arbitrarily chosen human V_H and one of two mouse λ chains as a germline repertoire is no barrier to generating very high-affinity IgG antibodies. This finding is consistent with work on the chicken immunoglobulin genes, where only a single functional V_H and V_L are utilized, but goes beyond this in two important respects. One is that chickens have somatically mutated antibodies prior to immunization (Weill and Reynaud, 1987; McCormack et al., 1991), thus allowing the argument that this sequence diversification takes the place of a large V region repertoire. Second, the germline V_H and $V\kappa$ genes utilized have been selected over many millions of years for their compatibility with many different antigens and thus they may represent the most polyfunctional $V_H V_L$ pair. In our system, we have chosen our $V_H V_L$ pair at random, suggesting that all or most such pairs are capable of binding a large number of different antigens, given a single, diverse V_H CDR3. This indicates that an antibody binding site excluding V_H CDR3 is exceptionally cross-reactive, at least until acted on by somatic mutation (Patten et al., 1996; Wedemayer et al., 1997). This interpretation is consistent with the observations that the residues characteristic of antibody binding sites have an unusually high proportion of asparagines, lysines, and aromatic amino acids, more typical of the interior of a globular protein than to its surface and capable of making a wide variety of different contacts (Kabat et al., 1977; Janin and Chothia, 1990; Padlan, 1990; Mian et al., 1991). It also has some similarities to the proposal of Pauling and others that a single antibody molecule could fold around different antigens in different ways to achieve specificity (Pauling, 1940).

The data presented here, as well as previous work, strongly suggest that the purpose of highly diverse CDR3 regions in all antigen receptors is to provide antigen specificity. It supports our earlier speculations (Davis et al., 1997, 1998) but is not consistent with the Protecton model of Cohn and colleagues, which argues that the primary antibody repertoire is solely defined by germline V_H and V_L gene sequences (i.e., CDR1 and CDR2) and that CDR3 is not important in the initial determination of antibody specificity (Langman and Cohn,

1987; Cohn and Langman, 1990). The Protecton model also argues that in the primary antibody repertoire, each specificity is expressed by a large number of B cells. Our data support the notion that certain antibody specificities may be relatively abundant prior to antigen stimulation in these mice. Thus, it would be interesting to test whether some CDR3 sequences are expressed at higher frequencies than others in the preimmune repertoire in the mice analyzed here and in wild-type animals.

The only deficit we have detected in the immune repertoire of mice with limited V gene(s) so far is in their inability to respond to bacterial polysaccharide antigens. Interestingly, chickens are unable to mount robust antibody responses to carbohydrates as well (Jeurissen et al., 1998), although this point is controversial (Granfors et al., 1982; Jalkanen et al., 1983). If the failure to respond to bacterial polysaccharides is due to the lack of appropriate V_H/V_L pair(s) to accommodate these molecules, the $HC1^{+/0}$ $IgH^{-/-}$ $Ig\kappa^{-/-}$ mice may have difficulties in clearing bacterial infections. The system described here will provide an experimental test for the idea that unique V_H/V_L pairs could be selected by pathogens during evolution and then fixed in the germline (Langman and Cohn, 1987; Cohn and Langman, 1990).

Experimental Procedures

Mice
B6 mice were originally obtained from the Jackson Laboratory (Bar Harbor, ME) and bred at our Animal Research Facility at Stanford University. Mice transgenic for the human heavy chain minilocus *HC1* were previously described (Taylor et al., 1992, 1994). Mice containing targeted deletions of the J_H region or the $J\kappa$–$C\kappa$ region were also described and are referred to in this paper as $IgH^{-/-}$ or $Ig\kappa^{-/-}$ (Chen et al., 1993a, 1993b). The *HC1* transgenic mice were bred onto the $IgH^{-/-}$ $Ig\kappa^{-/-}$ background. Details of mouse breeding and screening will be furnished upon request.

Reagents
BSA, CTB, CSA, DEX (MW 500,000), HEL, KLH, levan, OVA, PCC, and phOx were purchased from Sigma (St. Louis, MO). DNP-KLH and DNP-BSA were from Calbiochem (La Jolla, CA). TNP-Ficoll and TNP-BSA were from Biosearch Technologies, Inc. (Novato, CA). phOx-CSA and phOx-BSA were prepared as described (Mäkelä et al., 1978). PC-KLH and PC-BSA were kindly provided by Dr. Leonore A. Herzenberg (Stanford University).

Immunization, ELISA, and Hybridomas
Mice at 6–8 weeks of age were used in all experiments. To elicit immune responses, 100 μg CTB, HEL, KLH, PCC, OVA, DNP-KLH, or phOx-CSA in either complete Freund's adjuvant (for first injection) or incomplete Freund's adjuvant (for subsequent injections) was administered by intraperitoneal injection; 50 μg levan was administered in phosphate buffered saline (PBS) intraperitoneally without adjuvant; and 10 μg DEX, 10 μg TNP-Ficoll, or 100 μg PC-KLH (in PBS) was administered by intravenous injection. To detect *HC1*-encoded antibodies in the $HC1^{+/0}$ $IgH^{-/-}$ $Ig\kappa^{-/-}$ mice, diluted serum was added to microtiter wells coated with monoclonal mouse anti-human IgM (Clone CH6, The Binding Site, Birmingham, UK) or mouse anti-human IgG1 antibody (Clone HP6069, Fc specific, Calbiochem), and plate-bound *HC1* antibody was detected with alkaline phosphatase conjugated polyclonal goat anti-human IgM or goat anti-human IgG antibodies (μ chain and Fc specific, respectively, Sigma) followed by color development with p-nitrophenyl phosphate (Sigma 104); OD was measured at 405 nm on a microplate reader (Molecular Devices, Palo Alto, CA). Purified human serum IgM and human myeloma IgG1 (Sigma) were used as standards. Occurrence of antigen-specific antibodies after immunization was also examined by ELISA: plates were coated with 10–50 μg/ml of antigen, incubated with diluted serum, and antigen-bound human or mouse IgM or IgG was detected as above with alkaline phosphatase conjugated polyclonal goat anti-human (or mouse) IgM or goat anti-human (or mouse) IgG antibodies. To generate antigen-specific hybridomas, splenocytes were isolated from mice immunized with KLH, OVA, HEL, or DNP-KLH, and fused with the nonsecreting myeloma P3X63-Ag8.653 cells using standard procedures (Harlow and Lane, 1988). Hybridomas were subcloned by limiting dilution after being shown to produce antigen-specific IgM or IgG1 antibody in ELISA.

RT-PCR and Sequence Analysis
Total RNA was isolated from B cell hybridomas with the RNeasy mini kit (Qiagen). cDNA was synthesized using oligo $(dpT)_{12-18}$ (Collaborative Biomedical Products, Bedford, MA) and SUPERSCRIPT II RNase H⁻ reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) and amplified by PCR with TaqPlus Precision PCR system (Stratagene, La Jolla, CA). The primer pairs used in PCR were as follow: V_H5-51.L (5'-cctcgccctcctcctagctattc-3') and C_μ.1 (5'-gaaggaagtc ctgtgcgaggcag-3') (for μ chains); V_H5-51.L' (5'-ccatcctcgccctcctc cta-3') and C_γ1.1 (5'-cgcctgagttccacgacacc-3') (for γ1 chains); and $V_\lambda1,2.L$ (5'-tcacttatactctctctcctg-3') and $C_\lambda1,2,3.1$ (5'-ggaaggtg gaaaca[t/c/g]ggtga-3') (for λ chains). Amplified cDNA was cloned into pGEM-T Easy vector (Promega, Madison, WI) and sequenced with the Taq Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) on an Applied Biosystems 373A DNA Sequencer (PAN Facility, Stanford University).

Antibody Purification and Affinity Measurements
IgG1 antibody was purified from mouse serum or hybridoma supernatant by affinity column using goat anti-human IgG1 (Fc specific) antibodies conjugated to agarose beads (Sigma) according to standard procedures (Harlow and Lane, 1988), and exchanged into PBS using Centricon-30 (Amicon, Inc., Beverly, MA). Antigen or antibody was immobilized to the BIAcore CM5 chip (Pharmacia Biosensor) by standard amine chemistry. Binding of antibody to immobilized antigen was performed in PBS with 20 μl injections at a flow rate of 5 μl/min. Binding of HEL (freshly purified on a Superdex-75 size exclusion column [Pharmacia]) to immobilized antibody was carried out in PBS with 150 μl injections of protein at a flow rate of 100 μl/min. Regeneration of the biosensor surface was carried out with two 20 s pulse of 0.1 M glycine (pH 2.5) at a flow rate of 15 μl/min. All SPR measurements were performed at 25°C on BIAcore 1000. The association and/or dissociation phases of antigen-antibody interaction were fit with the single-site binding model of BIAEvaluation 3.0 (Pharmacia Biosensor).

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